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Isolation of a Precursor and a Nascent Chain Form of Glucose-6-Phosphate Dehydrogenase from Rat Uterus and Regulation of Precursor Processing by Estradiol

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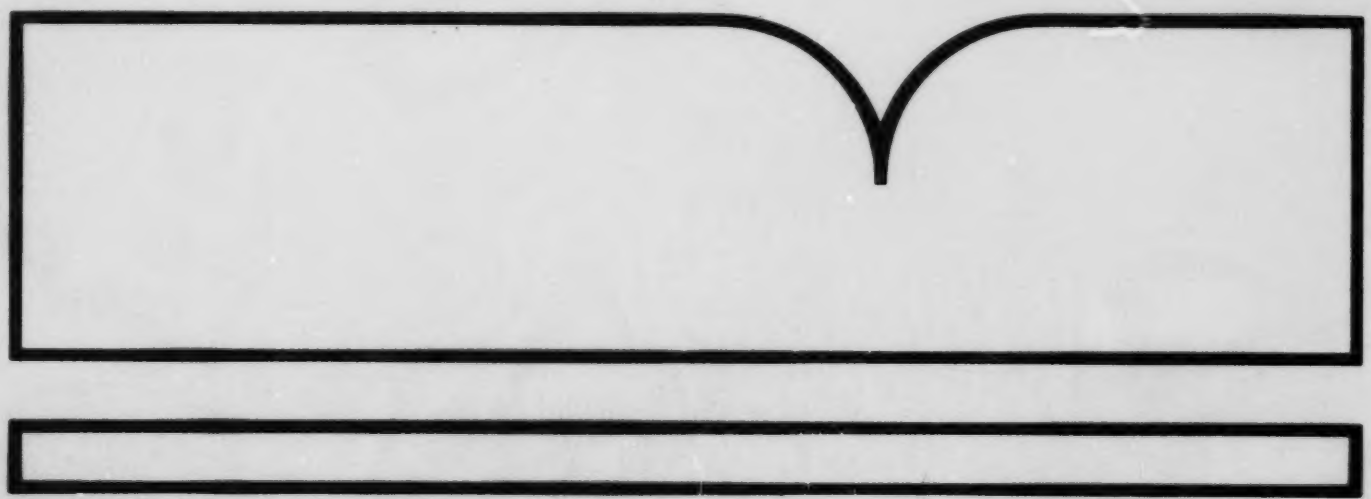

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16. ABSTRACT

SDS-polyacrylamide gel electrophoresis of anti-glucose-6-phosphate dehydrogenase immunoprecipitates from radiolabeled uterine tissue extracts previously revealed three proteins: A, B and C, which were tentatively identified as a 60–64 kDa precursor form, a 57 kDa predominant form, and a 40–42 kDa nascent peptide form of the enzyme, respectively. A peptide-mapping technique was used to examine structural homologies among A, B and C. Following the labeling of uterine proteins with [^{35}S]methionine, labeled proteins A, B and C were isolated by immunoprecipitation and electrophoresis. Each protein was individually co-digested with authentic, [^{3}H]methionine-labeled glucose-6-phosphate dehydrogenase using papain, the resulting peptides were resolved by isoelectric focusing and the peptides from the two sources on each gel were compared using double-label counting methods. Proteins A, B and C had at least eight peptides in common, both proteins A and C had two additional peptides in common that were not present in protein B, and B protein had two peptides that were either absent or present in reduced amounts in digests of proteins A and C. The extensive structural homology and immunoreactivity of these proteins indicated that proteins A, B and C were all related to glucose-6-phosphate dehydrogenase. The presence of two extra peptides in proteins A and C suggested that these peptides may be derived from a common NH_2-terminal leader sequence which was present in both the precursor and nascent peptide chains. The presence of two peptides that were present in protein B and absent from proteins A and C is easiest to explain if they are derived from the two ends of the molecule, with the corresponding peptides in proteins A and C containing additional peptide sequences that are 'normally' removed by endogenous proteolytic processing enzymes. Based on the relative time-course of synthesis of the three glucose-6-phosphate dehydrogenase-related proteins in control and estrogen-treated uteri, it appears that estradiol promotes an increase in the relative rate of transfer of label from protein A into B by stimulating the rate of processing of the precursor to the predominant form of the enzyme and enhances the rate of translational conversion of protein C into higher molecular weight forms.

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Isolation of a precursor and a nascent chain form of glucose-6-phosphate dehydrogenase from rat uterus and regulation of precursor processing by estradiol

Audrey M. Cummings * and Kenneth L. Barker **

Department of Biochemistry, Texas Tech University Health Sciences Center, Lubbock, TX 79430 (U.S.A.)

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Key words: Glucose-6-phosphate dehydrogenase; Protein processing; Peptide mapping; Estradiol; (Rat uterus)

SDS-polyacrylamide gel electrophoresis of anti-glucose-6-phosphate dehydrogenase immunoprecipitates from radiolabeled uterine tissue extracts previously revealed three proteins: A, B and C, which were tentatively identified as a 60–64 kDa precursor form, a 57 kDa predominant form, and a 40–42 kDa nascent peptide form of the enzyme, respectively. A peptide-mapping technique was used to examine structural homologies among A, B and C. Following the labeling of uterine proteins with [^{35}S]methionine, labeled proteins A, B and C were isolated by immunoprecipitation and electrophoresis. Each protein was individually co-digested with authentic, [^{3}H]methionine-labeled glucose-6-phosphate dehydrogenase using papain, the resulting peptides were resolved by isoelectric focusing and the peptides from the two sources on each gel were compared using double-label counting methods. Proteins A, B and C had at least eight peptides in common, both proteins A and C had two additional peptides in common that were not present in protein B, and B protein had two peptides that were either absent or present in reduced amounts in digests of proteins A and C. The extensive structural homology and immunoreactivity of these proteins indicated that proteins A, B and C were all related to glucose-6-phosphate dehydrogenase. The presence of two extra peptides in proteins A and C suggested that these peptides may be derived from a common NH_2-terminal leader sequence which was present in both the precursor and nascent peptide chains. The presence of two peptides that were present in protein B and absent from proteins A and C is easiest to explain if they are derived from the two ends of the molecule, with the corresponding peptides in proteins A and C containing additional peptide sequences that are 'normally' removed by endogenous proteolytic processing enzymes. Based on the relative time-course of synthesis of the three glucose-6-phosphate dehydrogenase-related proteins in control and estrogen-treated uteri, it appears that estradiol promotes an increase in the relative rate of transfer of label from protein A into B by stimulating the rate of processing of the precursor to the predominant form of the enzyme and enhances the rate of translational conversion of protein C into higher molecular weight forms.

Introduction

Identification of pyroglutamate as the NH_2-terminal amino acid of rat uterine and liver and human erythrocyte glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate:NADP$^+$ 1-oxidoreductase, EC 1.1.1.49) constituted the initial evi-

* Present address: MD-72, H.E.R.L., U.S. Environmental Protection Agency, Research Triangle Park, NC 27711, U.S.A.

** To whom reprint requests should be addressed.

Abbreviations: TEMED, *N,N,N',N'*-tetramethylethylenediamine; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis(2-(5-phenyloxazolyl))benzene.

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dence suggesting the existence of a precursor molecule [1,2]. Since methionine initiates the synthesis of proteins in eukaryotic systems, either the NH_2-terminal methionine or a methionine-containing peptide must be cleaved from a precursor to form the principal glucose-6-phosphate dehydrogenase monomer found in uterine cells. Further evidence came from experiments in which SDS-polyacrylamide gel electrophoresis gels of immunoprecipitates of radiolabeled uterine cytosols, prepared with an antiserum that was specific for rat glucose-6-phosphate dehydrogenase, showed that the label was first incorporated into two immunoreactive proteins of 60–65 and 40–42 kDa, respectively, and then into the predominant form of the enzyme of 57 kDa. The kinetics of labeling and the finding that the largest and smallest immunoprecipitable proteins could be extracted from purified uterine ribosomes suggested that the proteins may be precursor (60–65 kDa), nascent chain (40–42 kDa) and the authentic or predominant (57 kDa) forms of the enzyme, respectively [1].

The time required to translate the messenger RNA for uterine glucose-6-phosphate dehydrogenase has been estimated by determining mRNA transit times, and the time for enzyme processing was estimated as the lag interval between the initial time of release of an immunoprecipitable enzyme from the ribosomes and the time required for the immunoreactive protein to acquire pyroglutamate at its NH_2-terminus [3]. The observation that labeled, immunoprecipitable glucose-6-phosphate dehydrogenase which did not contain pyroglutamate on its NH_2-terminus was found after 2.5–9 min labeling time was considered as further evidence for the existence of a short-lived precursor molecule.

The purpose of the present study was to isolate the three forms of immunoprecipitable uterine glucose-6-phosphate dehydrogenase and to determine whether the three forms of the enzyme could be verified to be related to glucose-6-phosphate dehydrogenase based on peptide structural homologies. The time-course of synthesis in organ culture of the putative glucose-6-phosphate dehydrogenase precursor and nascent chain, relative to the predominant form of the enzyme, was also examined in uteri from control and estrogen-treated rats to further evaluate hormonal regulation of the translation and processing of this enzyme. It will be shown that the three proteins which are precipitated by antiserum to glucose-6-phosphate dehydrogenase contain homologous peptides and the relative rate of conversion of the high molecular weight precursor form of the enzyme into the predominant form of the enzyme is regulated by estradiol. This work was presented in part at the 7th International Congress of Endocrinology, Quebec, Canada, 1984. Excerpta Medica, Int. Congr. Series 652, Abstr. 441.

Experimental procedures

Animals. Mature female albino rats (Small Animal Supply Co., Omaha) weighing 160–180 g were ovariectomized 3 weeks prior to use. Animals were routinely fed Purina Lab Chow and water ad libitum. Estradiol (10 μg per rat) was administered by tail vein injection in 0.5 ml 5% ethanol/0.15 M NaCl.

Reagents. Substrates for the assay of glucose-6-phosphate dehydrogenase activity, 17β-estradiol, papain, acrylamide and TEMED were obtained from Sigma. Modified Eagle's medium and Earle's balanced salt solution were purchased from Grand Island Biological Company. Ampholenes for isoelectric focusing were obtained from Bio-Rad. Pansorbin® and goat anti-rabbit γ-globulin were purchased from Calbiochem. Radioactive materials, including L-[^{35}S]methionine (1000–1500 Ci/mmol) and L-[*methyl*-^{3}H]methionine (87–90 Ci/mmol), and NCS, were obtained from Amersham. The anti-glucose-6-phosphate dehydrogenase reagents used in this laboratory were made by immunizing rabbits with purified enzyme from rat liver. Purification of the enzyme, immunization and standardization of the antiserum was as previously described [1,6]. The anti-glucose-6-phosphate dehydrogenase was used as either the whole rabbit serum or as a protein-specific immunoglobulin preparation (immunoglobulin monospecific for glucose-6-phosphate dehydrogenase) that was prepared as follows. Electrophoretically pure rat liver glucose-6-phosphate dehydrogenase (more than 99.8% of protein on an SDS-polyacrylamide gel) was added to the antiserum at the immunological equivalence point in a volume of antibody buffer (0.05 M sodium phos-

phate (pH 7.0)/0.1 M sodium chloride/7.0 mM 2-mercaptoethanol/0.1 mM EDTA/0.1 mM NADP) equal to the volume of serum and a final concentration of 0.5% Triton X-100. The immunoprecipitate was sedimented through 0.5 M sucrose/0.3 M NaCl/0.5% Triton X-100, followed by washing the precipitate three times in the NaCl/Triton solution. The antibody-antigen complex was dissociated in 0.2 M glycine-HCl (pH 2.2) at 0°C for 3 h followed by a 1:10 dilution with cold water and adjustment of the pH to 7.0 by addition of 0.5 M Na_2HPO_4. One-tenth volume of swelled DEAE-cellulose equilibrated in antibody buffer diluted 1:4 with water was added and after 30 min, the DEAE-cellulose, to which the glucose-6-phosphate dehydrogenase was bound, was collected on a Buchner funnel. The immunoglobulin in the eluate was precipitated by adding solid ammonium sulfate to 50% saturation and centrifugation at $10000 \times g$ for 30 min. The precipitate was dialyzed against antibody buffer, titrated and frozen in aliquots. Whole serum had an equivalence point of 25 enzyme units (1 μmol NADP/min at 25°C) per ml (1.4 units/mg of γ-globulin) and the monospecific immunoglobulin had an equivalence point of 173 units/ml (without) or 393 units/ml (with addition of either Pansorbin® or goat anti-rabbit γ-globulin) corresponding to specific activities of 173 and 393 units/mg of γ-globulin, respectively.

Organ culture. Uterine proteins were labeled by incubating uteri in organ culture using a modification of a previously described system [4]. Two uteri were incubated in each flask containing either 4 ml of modified Eagle's medium (18-h incubations) or 2.0 ml of Earle's balanced salt solution supplemented with all amino acids contained in the complete medium except methionine and 0.35 g glucose/100 ml (2-h incubations). Both sets of media were supplemented with 1% bovine serum albumin, $2 \cdot 10^{-8}$ M estradiol, $1 \cdot 10^{-7}$ M dexamethasone and 0.05 mg/ml gentamycin. Each flask contained either [^{3}H]methionine or [^{35}S]-methionine at 0.05 mCi per uterus. Whole uteri were trimmed free of fat and mesentery and deeply nicked (crosswise) at approx. 2 mm intervals along both uterine horns prior to incubation. For the time-course experiments, media were prepared as for the 2-h experiment with two modifications: (1) all flasks contained 0.2 mCi [^{3}H]methionine (0.1 mCi/uterus) and (2) estradiol was omitted from the flasks containing control uteri. Following a 1 h preincubation of uteri in culture media at 37°C, the isotope was added and the flasks were returned to 37°C. At predetermined intervals, the uteri were removed from the flasks, rinsed in cold media and rapidly frozen in liquid nitrogen.

Enzyme preparation. Crude and fractionated preparations of uterine glucose-6-phosphate dehydrogenase were assayed at 25°C as previously described [6]. Glucose-6-phosphate dehydrogenase was partially purified from uterine cytosol using a modification of the method of Smith and Barker [6]. Uteri were homogenized with a Polytron homogenizer (Brinkman) in antibody buffer, centrifuged at $15000 \times g$ for 30 min and following centrifugation of the post-mitochondrial supernatant for 1 h at $200000 \times g$, the supernatant was precipitated with ammonium sulfate at 50% saturation and dialyzed overnight against antibody buffer. A ribosomal fraction was obtained by homogenization of uteri in a modified antibody buffer containing 5 mM $MgCl_2$ and no EDTA, centrifugation of the post-mitochondrial supernatant for 1 h at $200000 \times g$, and dissociation of proteins from the ribosomal pellet with EDTA, deoxycholate and Triton X-100 in complete antibody buffer. For the time-course experiments, the initial supernatant prepared in antibody buffer was dialyzed, heated rapidly to and maintained at 45°C for 5 min, and then was centrifuged for 1 h at $200000 \times g$ to prepare a supernatant from which all three forms of the enzyme could be immunoprecipitated. In all experiments, the term 'cytosol' represents an operationally defined fraction which excludes the major subcellular organelles but is likely to contain nascent peptides that were freed from the ribosomes upon homogenization in buffers containing EDTA.

Glucose-6-phosphate dehydrogenase was immunoprecipitated from cytosolic and ribosomal fractions of uteri as previously described [6] using an antiserum that was specific for the rat liver enzyme. In some experiments, complete immunoprecipitation of putative nascent or precursor forms of the enzyme was achieved by addition of Pansorbin® (heat-killed and formalin-hardened *Staphylococcus aureus* cells) to precipi-

tate all immunoglobulins including those specific for and bound to glucose-6-phosphate dehydrogenase. For these experiments, a monospecific anti-glucose-6-phosphate dehydrogenase rabbit immunoglobulin preparation was used to avoid overloading electrophoretic gels with non-specific immunoglobulin that would also be precipitated by the Pansorbin®. A similar profile of radioactivity on SDS-polyacrylamide gel electrophoresis gels was obtained with or without Pansorbin® except that Pansorbin® enhanced the yield of the higher and lower molecular weight immunoreactive peptides. Glucose-6-phosphate dehydrogenase from cytosols of uteri incubated for 18 h was isolated by immunoprecipitation in the absence of Pansorbin® since the predominant form of the enzyme can be completely precipitated by adding antibody at a slight (1.25-fold) excess. In the time-course experiments, glucose-6-phosphate dehydrogenase was immunoprecipitated using a monospecific antiserum, prepared from the rabbit antiserum specific for the enzyme, in conjunction with a goat anti-rabbit γ-globulin. Samples were incubated for 5 h at 0°C after the addition of monospecific antiserum alone. Following addition of the second antibody, the samples were incubated for 18 h at 0°C. Subsequently, the samples (approx. 2 ml) were layered over 0.5 M sucrose (0.5 ml) containing 0.5% each Triton X-100 and deoxycholate and centrifuged at $5000 \times g$ for 30 min prior to the washing steps.

The washed immunoprecipitates were dissociated in a dissociation buffer (0.1 M Tris (pH 10.5)/0.1 M dithiothreitol/1% SDS/6 M urea) and subjected to SDS-polyacrylamide gel electrophoresis according to the method of Laemmli [7]. Radioactivity in gel slices (2 mm) corresponding to the authentic or predominant glucose-6-phosphate dehydrogenase monomer (57 kDa), the putative precursor (60–65 kDa) and a putative major nascent chain form of the enzyme (42 kDa) was determined after solubilization of proteins in the gel slices with NCS containing 10% 4 M ammonium hydroxide. Where indicated, gel slices containing specific proteins were cut from identical gels and eluted for 8 h in collection cup buffer containing detergent (collection cup buffer: 0.01 M Tris-acetate (pH 8.6)/0.01% SDS; reservoir buffers 0.1 and 0.05 M Tris-acetate (pH 8.6)) using an ISCO electrophoretic sample concentrator (Model 1750). A portion of each eluted protein was re-assayed by SDS-polyacrylamide gel electrophoresis to ascertain homogeneity.

Co-digestion and peptide mapping. Aliquots of the [^{3}H]methionine-labeled authentic glucose-6-phosphate dehydrogenase monomer (approx. 40 000 dpm) were combined with aliquots of the [^{35}S]methionine-labeled enzyme monomer, putative precursor, and the putative major nascent chain form (peaks B, A and C from the SDS-polyacrylamide gel electrophoretic gels, respectively), each containing approx. 20 000 dpm of radioactivity. Each combination was digested with papain at a concentration of 1.67 mg/ml for 8 h at 55°C in 0.1 M Tris-acetate buffer (pH 8.6). Residual SDS from the dissociation and elution of the enzyme was found not to inhibit papain digestion of glucose-6-phosphate dehydrogenase as was previously described [8]. Papain was judged by preliminary experiments to be superior to other proteinases under the conditions used. The most susceptible points of peptide cleavage by papain in dilute SDS are at peptide bonds formed by the carboxyl groups of α-amino-substituted lysine and arginine, with minor cleavage points adjacent to leucine and glycine. The generation of peptides of identical isoelectric points following site-specific digestion by papain is considered to suggest amino acid sequence homologies between peptides from related proteins; however, in the absence of sequence data, these are referred to in this paper as structural homologies.

Following lyophilization, the peptides generated by co-digestion were dissolved in isoelectric focusing electrophoresis lysis buffer (9.5 M urea, 5% Nonidet P-40, Bio-Rad ampholenes: 1.6% pH 5–7 and 0.4% pH 3–10, and 5% 2-mercaptoethanol) and separated on 0.6 cm diameter × 10.5 cm long isoelectric focusing tube gels by a modification of a previously described procedure [9]. Modifications to the technique were: (1) the substitution of 0.1 M acetic acid for the 0.01 M phosphoric acid anode solution, (2) the use of the following ampholenes: 0.2 ml of pH 4–6, 0.2 ml of pH 5–7 and 0.1 ml of pH 3–10 per 10 ml of gel solution, and (3) the omission of the 1 h period electrophoresis at 800 V after 16 h of electrophoresis at 400 V. Slices (2 mm) of isoelectric focusing gels

containing the ^{35}S- and ^{3}H-labeled peptides were solubilized in NCS containing 10% 4 M ammonium hydroxide and then counted in a toluene fluor (4 g PPO, 100 mg POPOP and 1 liter toluene) using double-isotope techniques in a Beckman LS 7800 liquid scintillation counter.

Results

The strategy for determining structural homologies between various proteins that were immunoreactive with a specific anti-glucose-6-phosphate dehydrogenase antiserum was to label the predominant form of the immunoreactive enzyme with [^{3}H]methionine and the minor immunoreactive protein with [^{35}S]methionine under in vitro organ culture conditions and determine the electrophoretic similarities between methionine-containing peptides produced by site-specific cleavage of the proteins with papain. Rat liver and presumably rat uterine glucose-6-phosphate dehydrogenase contain 13 methionine residues per subunit which should yield a relatively uncomplicated electrophoretic profile of radioactive peptides [1]. The use of different isotopes for the reference and test peptides, respectively, allows simultaneous digestion of both samples in the same reaction mixture and analysis of the generated peptides on the same electrophoretic gel using double-label counting methods. [^{3}H]Methionyl-glucose-6-phosphate dehydrogenase was isolated by immunoprecipitation of the protein from uterine cytosol following an 18 h incubation of uteri in an organ culture medium containing [^{3}H]methionine. The SDS-polyacrylamide gel electrophoretic profile of the labeled immunoprecipitate indicates that the majority of the [^{3}H]-labeled protein (peak B) on the gel was the authentic, predominant form of glucose-6-phosphate dehydrogenase of 57 kDa that was observed previously [6] (Fig. 1). This protein was eluted from slices 18–20 of gels identical to that represented in Fig. 1 and was used in subsequent co-digestion studies to represent the authentic enzyme monomer.

[^{35}S]Methionine-labeled proteins corresponding to anti-glucose-6-phosphate dehydrogenase-immunoreactive proteins A, B and C were isolated from uterine cytosol following a 2 h incubation of uteri in an organ culture medium containing

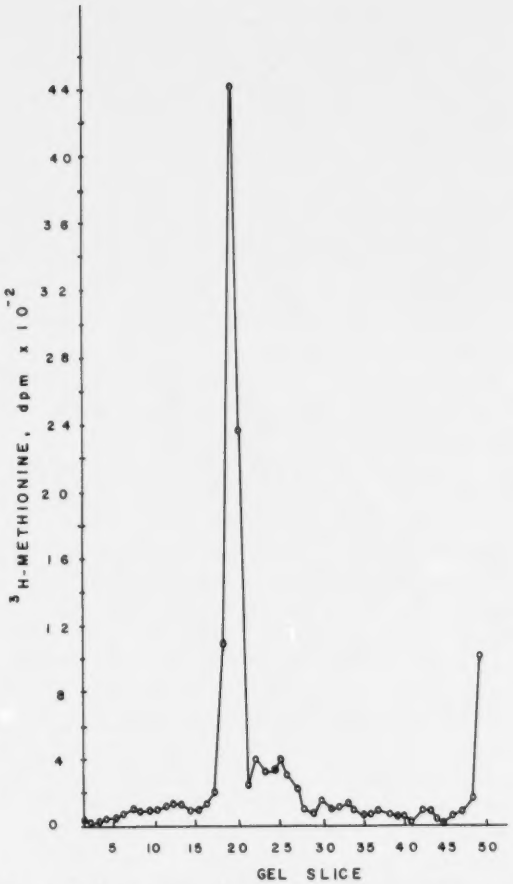

Fig. 1. SDS-polyacrylamide gel electrophoretic separation of labeled anti-glucose-6-phosphate dehydrogenase immunoprecipitable proteins from in vivo estradiol-treated rat uteri after an 18 h incubation of uteri in organ culture medium containing [^{3}H]methionine. Uterine supernatant fraction was prepared by centrifugation of the post-mitochondrial supernatant at 200000 × *g* for 1 h and contains released proteins and nascent peptides which are released from ribosomes due to the presence of EDTA in the homogenization buffer. Immunoprecipitation was completed without the addition of Pansorbin®. Radioactivity at gel slice 50 is free [^{3}H]methionine.

[^{35}S]methionine, using the Pansorbin®-assisted immunoprecipitation method (Fig. 2). One extra spike of radioactivity was seen between protein B and protein C and this spike of 50 kDa was found to be a minor contaminant, perhaps [^{3}H]-labeled immunoglobulin heavy chain produced by plasma

cells which were trapped in the tissue, which was capable of binding to Pansorbin® in the absence of added immunoglobulin. To minimize contamination of the immunoreactive proteins B and C with this extra protein, only the leading and trailing edges of peaks B and C, respectively, were eluted from identical gels. These three peaks are the ones that had been previously identified as the putative 60–64 kDa precursor (protein A), the authentic, predominant 57 kDa (protein B) and the putative 40–42 kDa nascent chain (protein C) forms of immunoreactive glucose-6-phosphate dehydrogenase, respectively [1,3]. After elution, portions of each protein sample from peaks A, B and C were re-analyzed on SDS-polyacrylamide electrophoresis gels (Fig. 3). Each of the three [^{35}S]methionine-containing proteins was found to be essentially free of extraneous protein and ran independently on SDS gels. Recoveries of radioactive proteins from gels containing proteins A, B and C by the electroelution method were 77, 81 and 90%, respectively.

The SDS-polyacrylamide gel electrophoretic profile of isotope-labeled proteins from an EDTA and detergent dissociated ribosomal fraction of the rat uterine homogenate was similar to that previously reported for these proteins after labeling in vivo with [^{3}H]glutamate [3] (data not shown). The enrichment of the amount of immunoreactive proteins A and C relative to B in the ribosomal

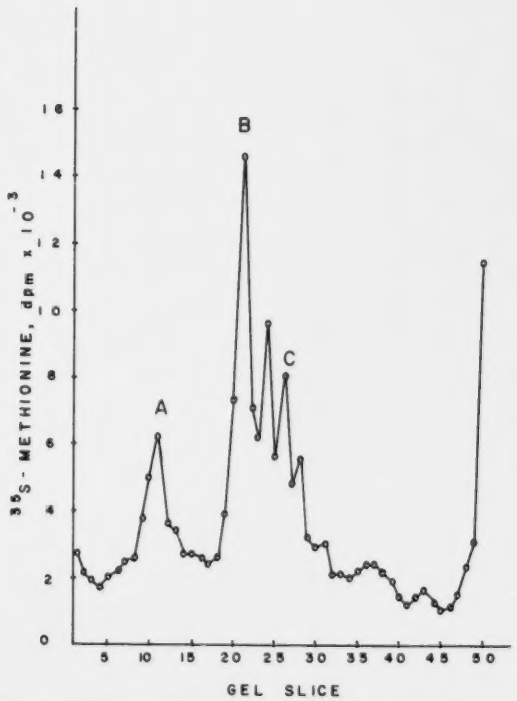

Fig. 2. SDS-polyacrylamide gel electrophoretic separation of labeled anti-glucose-6-phosphate dehydrogenase immunoprecipitable proteins from in vivo estradiol-treated rat uteri after a 2 h incubation in organ culture medium containing [^{35}S]methionine. Immunoprecipitation was assisted by addition of Pansorbin®. Peak B represents the authentic or predominant form of glucose-6-phosphate dehydrogenase, and peaks A and C represent the putative precursor and putative nascent peptide forms of the enzyme, respectively. The peak of radioactivity between peaks B and C was subsequently found to be a protein which interacts directly with Pansorbin® and was excluded during subsequent elution of protein from gel slices.

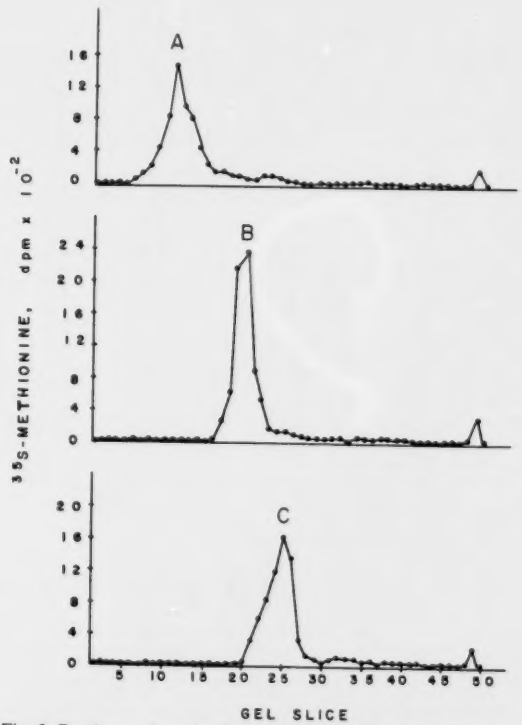

Fig. 3. Re-electrophoresis of anti-glucose-6-phosphate dehydrogenase-related proteins A, B and C after their electroelution and concentration from groups of gels identical to that represented in Fig. 2 and then re-electrophoresed separately on SDS gels.

fraction as compared with the cytosolic fraction was considered consistent with the previous tentative identification of proteins A and C as being the precursor and nascent forms of glucose-6-phosphate dehydrogenase, respectively. It further suggested that the putative precursor may in fact be either a large nascent protein or a completed protein that remains tightly bound to the ribosome fraction.

The profile of radioactivity, on a single isoelectric focusing gel, of the peptides which were generated by papain co-digestion of 'authentic' [^{3}H]-

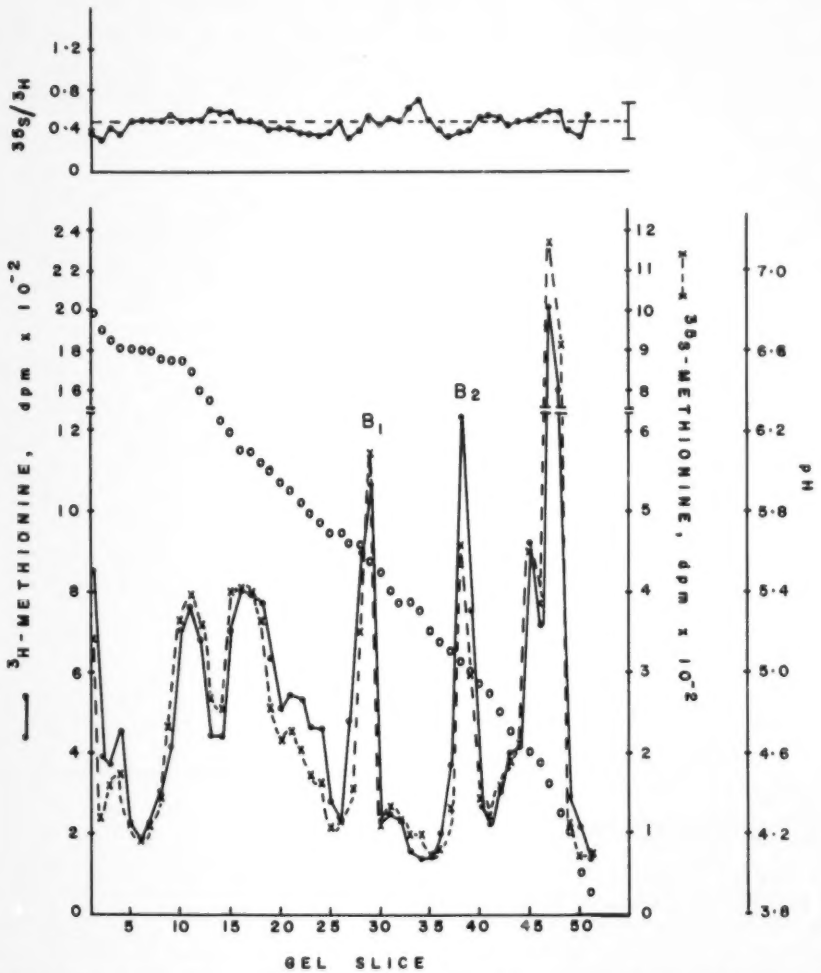

Fig. 4. Radioactive methionine-containing peptides generated by papain co-digestion of the predominant uterine form of authentic [^{3}H]methionyl-glucose-6-phosphate dehydrogenase with [^{35}S]methionine-labeled protein B after separation on an isoelectric focusing electrophoresis gel. Radioactivity of peptides generated from the major [^{3}H]methionine-labeled protein (●-●) extracted from gels identical to that described in Fig. 1 and from [^{35}S]methionine-labeled protein B (×--×) which is as characterized in Fig. 3 (middle panel) and the pH gradient (●) are given in the lower panel and the $^{35}S/^3H$ ratios of individual gel slices are given in the upper panel. The dashed line in the upper panel is the mean of the $^{35}S/^3H$ ratio for all gel slices and the vertical bar represents two standard deviations above and below that mean ($\bar{x} = 0.47$; S.D. = 0.087). This was considered to be the range of ratios beyond which an individual peptide was considered to be significantly different from the authentic enzyme as given in Figs. 5 and 6.

methionyl-glucose-6-phosphate dehydrogenase (protein B from 18-h-labeled uterine cytosol) with [^{35}S]methionyl protein B from 2-h-labeled uterine cytosol is given in Fig. 4. Ten identical peptides with isoelectric points from p*I* 4.2 to 6.7 were generated by papain digestion of protein B from the two sources. The peptides were at identical positions on the gels and the relative amount of radioactivity between individual peptides was the same. These data demonstrate extensive structural homology between the [^{3}H]methionine-labeled glucose-6-phosphate dehydrogenase generated from longer labeling times and protein B from the 2-h [^{35}S]methionine-labeled uteri indicating that

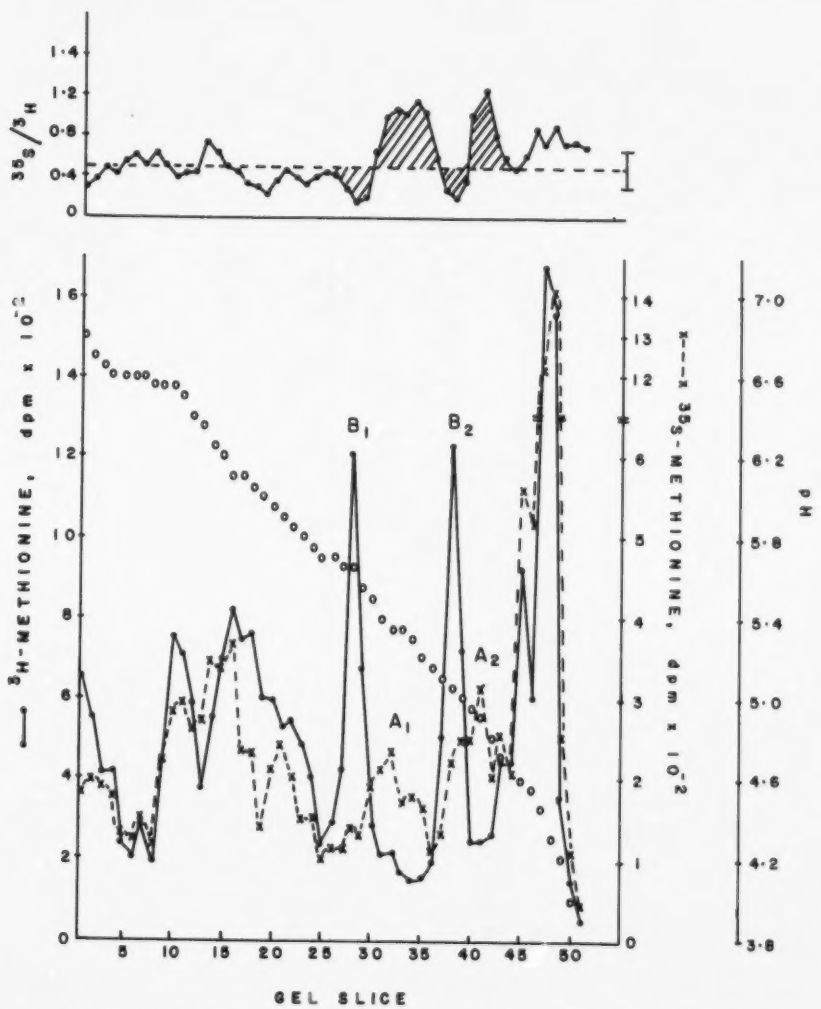

Fig. 5. Radioactive methionine-containing peptides generated by papain co-digestion of the predominant uterine form of authentic [^{3}H]methionyl-glucose-6-phosphate dehydrogenase with [^{35}S]methionine-labeled protein A after separation on an isoelectric focusing electrophoresis gel. The symbols are as given in Fig. 4 except that (●-●) represents protein A which was characterized in Fig. 3 (upper panel).

protein B produced by the shorter labeling interval was equivalent to the authentic enzyme. Since the [^{35}S]methionyl protein B was added at half the amount of radioactivity as the [^{3}H]methionyl-glucose-6-phosphate dehydrogenase, the $^{35}S/^{3}H$ ratio should be 0.5. The ratio was nearly 0.5 in every gel slice, indicating homology throughout. The mean and standard deviation of the ratios for all gel slices was 0.47 ± 0.087. From this, it was considered that ratios in individual gel slices which deviated from the mean by more than 0.174 (two standard deviations) would indicate a significant

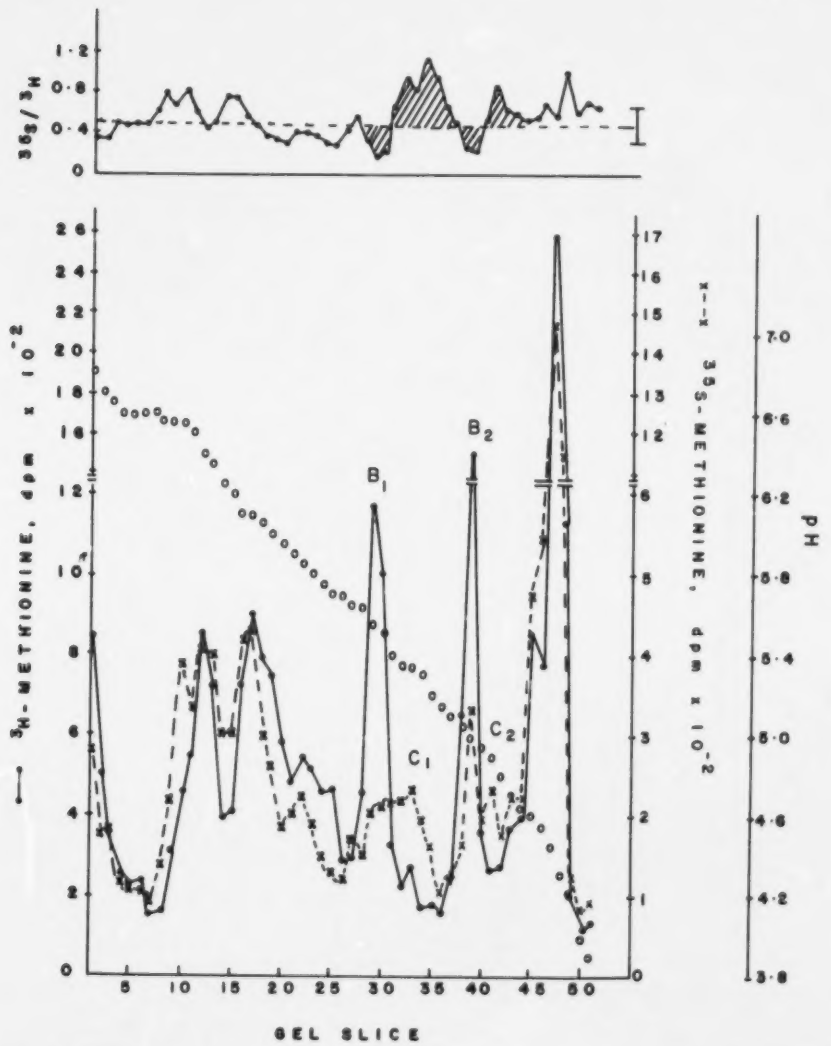

Fig. 6. Radioactive methionine-containing peptides generated by papain co-digestion of the predominant uterine form of authentic [^{3}H]methionyl-glucose-6-phosphate dehydrogenase with [^{35}S]methionine-labeled protein C after separation on an isoelectric focusing electrophoresis gel. The symbols and descriptions are as given in Fig. 4 except that (●-●) represents protein C which was characterized in Fig. 3 (lower panel).

deviation from structural homology by an individual peptide.

The profile of radioactivity, on an isoelectric focusing gel, of the peptides derived from the papain co-digestion of the 'authentic' [^{3}H]-methionyl-glucose-6-phosphate dehydrogenase with [^{35}S]methionyl protein A (the higher molecular weight putative precursor of glucose-6-phosphate dehydrogenase) from cytosol is given in Fig. 5. Six major and two minor radioactive peptides were derived from both protein A and from authentic glucose-6-phosphate dehydrogenase, indicating a significant structural homology between the two proteins. Two peptides, designated B_1 and B_2 were either not present (B_1) or were present in a reduced amount (B_2) in protein A and two peptides, A_1 and A_2, were present in protein A but not in the authentic glucose-6-phosphate dehydrogenase. The $^{35}S/^3H$ ratios deviated from 0.5 by more than two standard deviations (calculated from Fig. 4) in those slices corresponding to peptides A_1, A_2, B_1 and B_2, which indicated lack of structural homology of peptides in those areas.

The profile of radioactivity in the peptides generated by the papain co-digestion of authentic glucose-6-phosphate dehydrogenase and protein C following separation on an isoelectric focusing gel is given in Fig. 6. Again six major and two minor peptides were present in both proteins B and C, indicating significant structural homology. Two peptides in protein C, C_1 and C_2, were absent from authentic glucose-6-phosphate dehydrogenase and two peptides in authentic glucose-6-phosphate dehydrogenase, B_1 and B_2, were either not present or were in a reduced amount (B_2) in protein C. The deviation of the $^{35}S/^3H$ ratio from 0.5 in the areas of the gel corresponding to peaks C_1, C_2, B_1 and B_2 indicated the lack of structural homology in those areas. With concern for the possibility that protein C may have contained traces of the Pansorbin®-absorbed contaminating protein which migrated on the initial electrophoretic gels between protein B and C and that peptides C_1 and C_2 might be derived from the contaminating protein, an aliquot of the contaminating protein was prepared and co-digested with authentic enzyme. No structural homology was observed between any peptides seen in either protein B or in the region of peptides C_1 and C_2. The extra peptides, A_1 and A_2 in protein A and C_1 and C_2 in protein C, have nearly the same relative isoelectric points, suggesting that proteins A and C may both contain additional common peptides that are not present in protein B. Together, these results establish that major amino acid structural homologies exist between proteins A, B and C in addition to their shared immunoreactivity with antiserum that is specific for rat glucose-6-phosphate dehydrogenase.

Undigested [^{35}S]methionine-labeled proteins A, B and C isolated from 2-h-labeled uterine cytosol were run separately on isoelectric focusing gels and the profiles of radioactivity are given in Fig. 7. The results indicate that the major peaks of radioactivity in the undigested proteins do not coincide with any of the peptide peaks seen following digestion with the possible exception of a small lesser peak adjacent to peptide A_1 (gel slice 34, Fig. 5). The nature of protein A may be such that conditions optimal for the complete digestion of proteins B and C could have permitted a trace of protein A to remain undigested. All three proteins had similar isoelectric points of from 5.1 to 5.2, under the conditions used. These values differ from the p*I* of 6.52 which was reported previously [1] and may be due to the use of sodium dodecyl sulfate in the isolation of the enzyme which was analyzed in the present study. Proteins A and C, which appeared to be slightly heterogeneous in Fig. 3, also showed some heterogeneity and deviation of their isoelectric points from that of protein B. The absence of significant amounts of proteins A, B and C on the isoelectric gels of papain-digested samples indicate that the papain digestion had been complete.

To evaluate the specificity of the antisera for proteins A, B and C and to rule out further the possibility that proteins A and C could be derived from the non-specific adherance of these proteins to Pansorbin®, the proteins were isolated by an alternate procedure. Uterine cytosols were prepared from tissues that had been incubated in a medium containing [^{35}S]methionine, using a complete antibody buffer (with EDTA) to which 1 mM phenylmethylsulfofluoronate (an inhibitor of serine proteinases) was added, and the immunoprecipitates were prepared using a monospecific antibody to the enzyme, with goat anti-

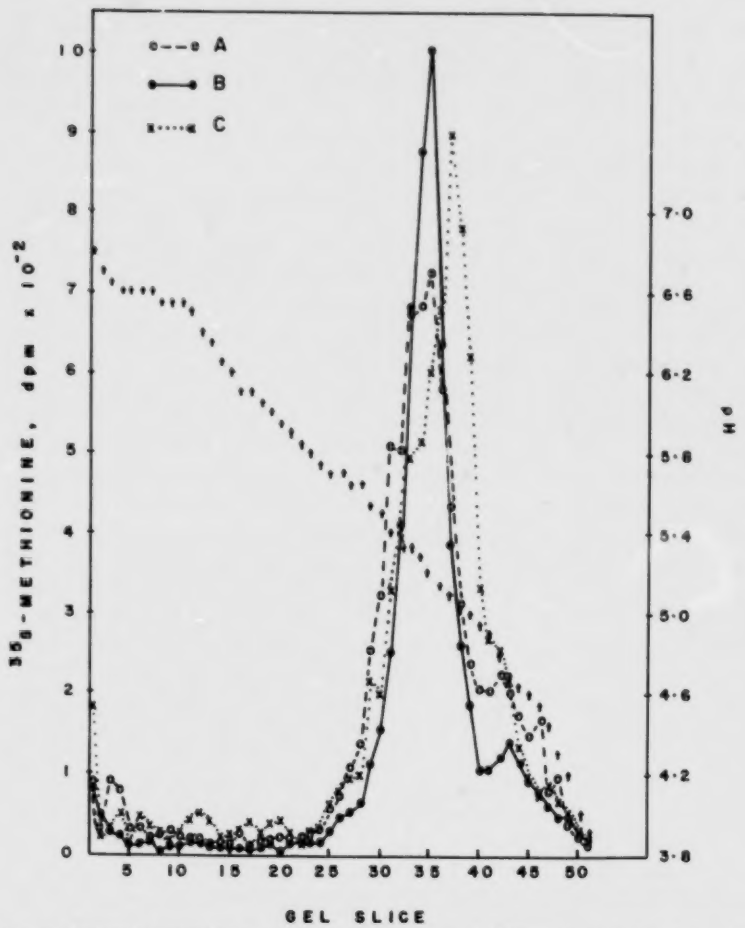

Fig. 7. Isoelectric separation of radioactivity of undigested, [^{35}S]methionine-labeled proteins A, B and C run on isoelectric focusing gels. The proteins were prepared and characterized as described in Fig. 3 and were subjected to isoelectric focusing as in Figs. 4–6 except that proteinase digestion was omitted. Symbols: + represents the pH gradient; ○, ● and × represent glucose-6-phosphate dehydrogenase-related proteins A, B and C, respectively.

rabbit γ-globulin instead of Pansorbin®, added to complete precipitation of proteins A and C. In addition, the immunoprecipitates were vigorously washed with 0.3 M NaCl after being centrifuged through 0.3 M NaCl which contained 0.5 M sucrose. The specificity of this immunoprecipitation reaction was evaluated by either preincubating the antisera with a 100-fold excess of unlabeled homogeneous rat liver glucose-6-phosphate dehydrogenase (which has the characteristics of protein B) or by addition of control rabbit γ-globulin or control preimmune rabbit serum. The results given in Fig. 8 indicate that the use of the second antibody technique, instead of Pansorbin®, gives similar molecular weight profiles of immunoreactive proteins to that seen in the first 30 gel slices of Fig. 2, and the appearance of all three proteins, A, B and C was eliminated by competition with homogeneous rat liver glucose-6-phosphate dehydrogenase or by substitution of control rabbit γ-globulin or preimmune rabbit serum for the monospecific antibody to glucose-

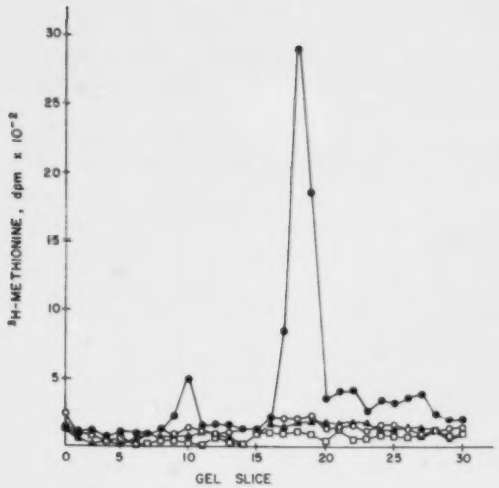

Fig. 8. SDS-polyacrylamide gel electrophoretic separation of anti-glucose-6-phosphate dehydrogenase immunoprecipitable proteins from [^{35}S]methionine-labeled rat uteri. Immunoprecipitates were prepared using monospecific anti-glucose-6-phosphate dehydrogenase, with (○) or without (●) preincubation of the antibody for 30 min with 100 units of purified rat liver glucose-6-phosphate dehydrogenase, or using non-immune rabbit IgG (▲) or preimmune control rabbit serum (□). All immunoprecipitation reactions were completed by addition of anti-rabbit IgG.

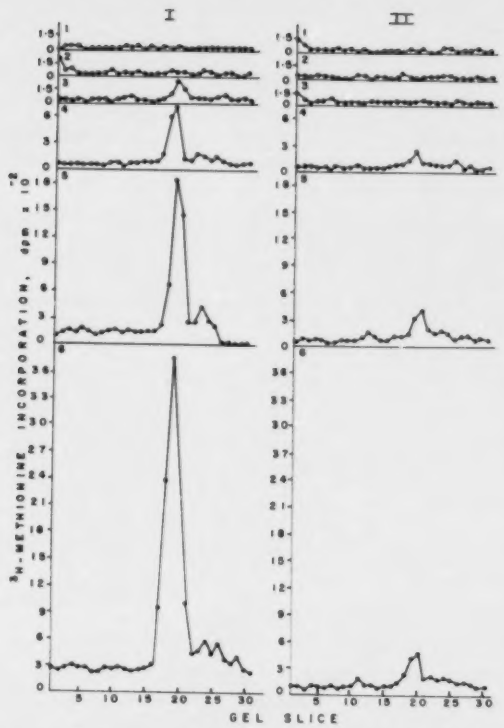

Fig. 9. Time-course of labeling of glucose-6-phosphate dehydrogenase in uteri from 16 h estradiol-treated (I) and control (II) rats. Graphs 1–6 represent 0, 2.5, 5, 10, 15 and 20 min of incubation of uteri in organ culture medium after addition of [^{3}H]methionine. Each graph represents the amount of [^{3}H]methionine incorporated into anti-glucose-6-phosphate dehydrogenase immunoprecipitates prepared by the monospecific anti-glucose-6-phosphate dehydrogenase/anti-rabbit IgG system followed by separation by SDS-polyacrylamide gel electrophoresis.

6-phosphate dehydrogenase. When proteins A, B and C were electroeluted from gels prepared as in Fig. 8, and then co-digested with [^{3}H]methionyl-glucose-6-phosphate dehydrogenase using papain, peptide maps identical to those seen in Figs. 4–6 were obtained (data not shown). Similarly when [^{35}S]methionine-labeled proteins A and C were prepared from EDTA and detergent extracts of uterine ribosomes and then co-digested with the [^{3}H]methionine-containing protein B, the same sequence homologies were seen as for proteins A and C obtained from cytosol (data not shown).

A comparison of the time-course of the in vitro labeling of the three anti-glucose-6-phosphate dehydrogenase-immunoreactive proteins in uteri obtained from control or estradiol-treated rats is given in Fig. 9. The data show that radioactivity was first incorporated into glucose-6-phosphate dehydrogenase, after 5 min of incubation, in tissues from estrogen-treated rats while the first incorporation of label into the enzyme occurred after 10 min of incubation in uteri from control animals. These data indicated that estradiol treatment induced the synthesis of uterine glucose-6-phosphate dehydrogenase. The hormonal regulation of precursor processing was also revealed upon further analysis of the data. Specifically, the proportion of label in fractions A, B and C relative to total label in immunoprecipitable glucose-6-phosphate dehydrogenase (Table I) revealed that the percent of total label present in protein A (the precursor) decreased and the percent of label in protein B (the authentic enzyme) increased with incubation time in uteri from estrogen-treated rats.

TABLE I

EFFECT OF ESTRADIOL AND TIME OF INCUBATION ON THE RELATIVE INCORPORATION OF [^{3}H]METHIONINE INTO GLUCOSE-6-PHOSPHATE DEHYDROGENASE A, B AND C AND ON THE AMOUNT OF LABELED PRECURSOR (A) AS COMPARED WITH THE AMOUNT OF LABELED AUTHENTIC ENZYME (B)

The data presented here are derived from Fig. 9. For each time point, the radioactivity in four gel slices from peaks A, B and C were totaled. The percent of radioactivity in each peak was calculated as A/total, B/total and C/total. Ratio of radioactivity in A vs. B was calculated as A/B.

Percent of total radioactivity	Treatment	Time of incubation (min)					
		0	2.5	5	10	15	20
A	estradiol	–	–	20	11	9	9
	control	–	–	–	20	20	20
B	estradiol	–	–	57	66	71	74
	control	–	–	–	50	52	50
C	estradiol	–	–	23	23	20	17
	control	–	–	–	30	28	30
Ratio A/B	estradiol	–	–	0.35	0.16	0.13	0.12
	control	–	–	–	0.39	0.39	0.39

By contrast, the proportion of label in both proteins A and B remained relatively constant with time in the control uteri during the interval studied. These data are consistent with the previous indication that estrogen promotes both the synthesis of glucose-6-phosphate dehydrogenase and the processing of the precursor (protein A) to the predominant form of the enzyme (protein B) [3]. Likewise, the percent of total radioactivity seen in protein C (the nascent chain of glucose-6-phosphate dehydrogenase which seems to accumulate due to a reduced rate of translation of codons which are reached when the protein reaches a M_r of 40000–42000) is also less for uteri from estradiol-treated rats than is seen in uteri from control rats. This is consistent with the previous suggestion that the 'read through' of this rate-limiting translation zone may be regulated by estradiol [3].

Discussion

Previously, SDS-polyacrylamide gel electrophoresis of anti-glucose-6-phosphate dehydrogenase immunoprecipitates prepared from uteri of estradiol-treated rats after short intervals of in vivo incorporation of radioactive amino acids revealed the presence of two proteins in addition to the predominant form of the enzyme which has a subunit M_r of 57000 in our hands [1,3]. One protein had an M_r of 60000–64000 and the other had an M_r of 40000–42000. We initially considered these extra proteins to be contaminants which were co-precipitated by entrapment with the immunoglobulin-enzyme complexes. Upon further analysis, it was observed that they were synthesized prior to the predominant form of the enzyme, their precipitation by antisera to glucose-6-phosphate dehydrogenase could be blocked by adding competitive levels of purified glucose-6-phosphate dehydrogenase and they could be extracted from uterine ribosomes if the initial tissue homogenates were prepared in the absence of EDTA, which promotes ribosome dissociation and release of nascent or partially completed proteins [1,3]. For these reasons, we proposed that protein A (60–64 kDa) was a high molecular weight precursor of the predominant form (protein B, 57 kDa) of the enzyme and that protein C (40–42 kDa) was a nascent chain form of the enzyme which accumulated due to a non-uniform rate of elongation of the nascent enzyme [3]. These tentative suggestions required evaluation of proteins A, B and C for amino acid sequence or structural

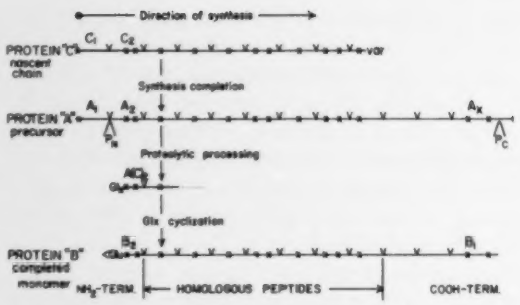

Fig. 10. Proposed scheme of cleavage of glucose-6-phosphate dehydrogenase-related proteins A, B and C by papain. v = papain site; * = probable methionine; P_N = NH_2-terminus proteolytic processing site; P_C = COOH-terminus proteolytic site; Glx = glutamate or glutamine; < Glu = pyroglutamate; var = variable but slow translation zone.

homologies. The results given in Figs. 4–6 indicate that proteins A and C contain a minimum of eight methionine-containing peptides in common with protein B, which contains 13 methionine residues per molecule [1], after complete digestion with papain. Five of the eight peptides in protein B are labeled to a greater extent than the other three, suggesting they may contain more than one methionine residue per peptide. This extensive structural homology suggests that the three proteins likely contain common amino acid sequences and supports the contention that they are related to the completed glucose-6-phosphate dehydrogenase monomer as previously proposed.

The three proteins are dissimilar, however, in the region of papain-produced peptides A_1, A_2, C_1, C_2, B_1 and B_2 from proteins A, C and B, respectively. Fig. 10 describes a scheme in which the dissimilarity could be explained if one assumes that proteins A, B and C are a high molecular weight precursor, a completely processed protein and a partially completed nascent chain of the glucose-6-phosphate dehydrogenase subunit, respectively. If analogous to the precursor of the light-chain of immunoglobulin [10] which, like glucose-6-phosphate dehydrogenase, has pyroglutamate on its amino terminus, both the unprocessed precursor and the nascent chain should contain a leader sequence on their NH_2-terminus which would give rise to at least one extra methionine-containing peptide relative to the processed enzyme upon hydrolysis by papain. This is consistent with the observations that the additional peptides derived from the precursor (A_1 and A_2), and the incomplete nascent protein (C_1 and C_2), respectively, have identical isoelectric points as would be expected if both proteins contained an identical NH_2-terminus leader sequence. A mechanism for the papain cleavage of protein B to generate the two unique peptides, B_1 and B_2, that are not present in protein A or C is also proposed. The extra peptides in the papain digest of protein B may be present on the two ends of protein B. B_1 could be generated by papain cleavage of a peptide from the COOH-terminus of protein B and this peptide would not be expected in protein A since it would not have been trimmed to a uniform length by an in vivo COOH-terminal processing enzyme. It would not be present in the shorter nascent protein C, since the incomplete protein would not yet contain the complete sequence of peptide B_1. Peptide B_2 is interesting in that it appears to be present in both proteins A and C, but in reduced amounts relative to the amount produced from protein B. This would be easiest to explain if peptide B_2 is derived from the pyroglutamate containing NH_2-terminus of protein B and if the NH_2-terminus processing enzyme has an amino acid sequence specificity that resembles that of papain. The incomplete production of peptide B_2 from proteins A and C after papain digestion could be due to only partial spontaneous cyclization of an NH_2-terminal glutamate or glutamine residue to produce pyroglutamate. We plan to evaluate peptide B_2 in tissues labeled with [^{3}H]glutamate for the presence of [^{3}H]pyroglutamate to verify this suggestion.

While NH_2-terminal processing of precursor peptides has been demonstrated for immunoglobulin light-chain and for human placental lactogen [10,11], evidence for COOH-terminal processing is less common. According to Planck et al. [12], during the binding and internalization of epidermal growth factor into fibroblasts, the protein is modified through sequential cleavage of amino acids from the COOH-terminus. Hook et al. [13] have described a carboxypeptidase obtained from bovine chromaffin granules that converts a labeled Met-enkephalin precursor to Met-enkephalin by the removal of COOH-terminal amino

acids. These previous general observations provide a precedent for our suggestion that both the NH_2- and COOH-termini of uterine glucose-6-phosphate dehydrogenase are processed in vivo. The processing sites of several prohormones, including pro-opiomelanocortin, pro-vasopressin, pro-oxytocin and the light-chain of insulin are reported to be at Lys-Arg, Lys-Lys or Arg-Arg residue pairs by an enzyme with trypsin-like specificity [14]. Since papain cleaves mainly at these basic amino acids, and since papain digestion of proteins A and C generates reduced amounts of one of the peptides (peptide B_2) found in protein B then one in vivo cleavage site of the precursor of glucose-6-phosphate dehydrogenase may be due to an enzyme with a specificity for these basic amino acid pairs.

The major significance of these data, which confirm the nascent chain-precursor-enzyme relationships among uterine glucose-6-phosphate dehydrogenase-related proteins C, A and B, respectively, lies in the identification of potential points of regulation by estradiol of the complete synthesis of the enzyme in the uterus. Previous work has shown that estradiol can increase uterine glucose-6-phosphate dehydrogenase mRNA activity [15] and decrease the translation time for the enzyme [3]. Also, based on a reduction in the time required for [^{14}C]pyroglutamate formation in immunoprecipitable glucose-6-phosphate dehydrogenase after incubation of tissue in [^{14}C]glutamate, it was suggested that the 'processing' of or removal of an NH_2-terminal peptide from a precursor form of glucose-6-phosphate dehydrogenase was also enhanced by estradiol [3]. In the time-course data presented in Fig. 9 and Table I, estradiol appears to increase the relative rate of conversion of protein A to B, since the proportion of the total radioactivity present in all forms of glucose-6-phosphate dehydrogenase tends to shift from the precursor to the predominant form of the enzyme after short intervals of labeling in the hormone-primed tissues. In the absence of estrogenic stimulation, a parallel rise in the proportion of label present in both proteins A and B is seen. These observations are consistent with the estrogenic regulation of the processing of the precursor to the predominant form of glucose-6-phosphate dehydrogenase. The results also suggest that in vivo processing is delayed, after synthesis, until the protein is substantially complete. Regulation of precursor processing by estrogen may be achieved by promoting the activation or synthesis of an enzyme which is required to cleave peptide bonds in pre-glucose-6-phosphate dehydrogenase to produce the mature enzyme in rat uterus. Data presented here provide some tentative clues to the specificity of at least one of the in vivo processing enzymes. Specifically, since papain does not generate peptide B_1 (which we propose is produced by a cleavage site at the COOH-terminus of the completed enzyme monomer) from protein A, it suggests that the in vivo processing event which gives rise to peptide B_1 does not occur at a site for which major or minor papain specificity exists. Thus, lysine or arginine residues are probably not at this processing site as they are for many proteins [14]. The in vivo cleavage site which we propose to be responsible for processing the NH_2-terminus of the enzyme may be 'partially' analogous to a papain-specific cleavage site. From these considerations and those previously discussed [3] we would predict a sequence of (Arg/Lys)–(Arg/Lys)-Glx-Gly at the NH_2-terminal cleavage site with cleavage being on the amino side of the glutamine residue by a trypsin-like enzyme that is commonly active in processing the basic amino acid groupings of several other proteins [14]. This sequence remains to be confirmed by either sequencing the precursor of rat uterine glucose-6-phosphate dehydrogenase or by sequencing a cDNA to the complete mRNA for rat glucose-6-phosphate dehydrogenase.

The estrogen-dependent accumulation of the nascent chain of glucose-6-phosphate dehydrogenase (protein C) suggests that the rate of translation of a specific sequence of amino acids encountered when the protein reaches a molecular weight of 42000 is regulated by the hormone. We have recently found that estradiol increases the availability of tRNAs in the uterus for selected amino acids in part by the repair of the 3′-CCA terminus of defective but repairable tRNAs [16]. Thus, estradiol may be regulating the rate of translation of sequences found in glucose-6-phosphate dehydrogenase by controlling the availability of rate-limiting amounts of certain tRNAs which are required to support translation of these regions.

Again, comparison of the composition of amino acids in the translation restriction zone with the selected tRNAs that are regulated by estradiol will give important clues regarding the mechanism of regulation of translation of this enzyme in the uterus by estradiol.

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